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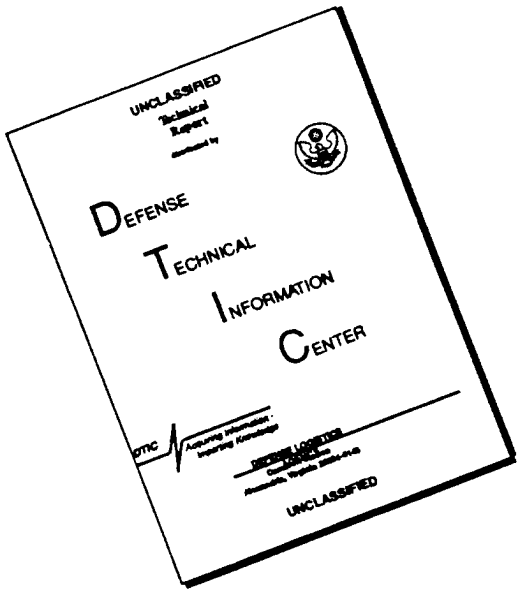

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Jennifer A Sardesai
Principal Investigator's Signature

1/21/96
Date

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SUMMARY STATEMENT OF ACCOMPLISHMENTS

The overall goal of this study is to compare the effects of tamoxifen and estrogen on the protein kinase C (PKC) pathway in osteoblastic cells. This will help us assess whether tamoxifen has estrogenic actions at the cellular level in bone. The first task of this study was to complete the determination concerning which PKC isozymes are expressed in osteoblasts. As a means of characterizing the expressed isozymes, we also examined their sensitivity to long-term phorbol ester treatment. A manuscript has been prepared based on this work and will be submitted for publication soon. This manuscript is presented below in the format requested by the U.S. Army Medical Research and Materiel Command Breast Cancer Research Program. The figures referred to in the text can be found in Appendix 1.

In addition, we have also begun experiments to examine the responsiveness of the PKC isozymes in osteoblasts to estrogen and tamoxifen (Task 2). Information from these studies is located in Appendix 2. We have not initiated the experiments set forth in Task 3 (determination of the effects of other sex steroids on PKC isozyme expression); we will begin these experiments in the near future.

INTRODUCTION

The protein kinase C (PKC) enzyme family consists of at least 11 isozymes with unique tissue distributions and substrate specificities (1-6). These isozymes comprise at least three classes, termed the conventional, novel, and atypical isozymes; a fourth class has also been proposed, the charter member being PKC-μ. The isozymes within a given class share common activation requirements due to structural features within that class. The conventional isozymes, PKC-α, -β_I, -β_{II}, and γ, require phosphatidylserine (PS), diacylglycerol (DAG), and calcium (Ca^{2+}) for activation. The novel isozymes, PKC-δ, -ϵ, -η, and -θ, also require PS and DAG for activation, but are Ca^{2+}-independent. The atypical isozymes, PKC-ζ, -ι/λ, require PS but are both Ca^{2+}- and DAG-independent. The activation requirements of PKC-μ are most like those of the novel isozymes, but several features separate μ from this class, including the presence of two unique hydrophobic amino-terminal domains in μ (5).

The common structural features of a given class of PKCs also confer a similar sensitivity of these isozymes to pharmacological agents such as phorbol esters. Phorbol ester-sensitive PKC isozymes are activated by acute phorbol treatment (7) but down-regulated with more prolonged exposures (8). Phorbol esters act as DAG analogs; therefore, the conventional and novel PKC isozymes, which bind DAG, also bind and are responsive to phorbol esters (2). In contrast, the atypical isozymes, which lack one of the two zinc fingers necessary to bind DAG, also fail to bind phorbol esters and are generally phorbol-insensitive (2,4).

PKC has been implicated in the signalling events associated with numerous cellular responses, including bone resorption. The role of PKC in bone remodeling is not clear, however. PKC activity is increased in particulate fractions from parathyroid hormone (PTH)-treated bones or bone cells (9-12), whereas antagonists of PKC prevent PTH- and calcitriol-stimulated resorption (13,14). In different studies, phorbol esters have been found to stimulate bone resorption (15-17) and to inhibit resorptive responses to PTH (18).

Little is known about specific PKC isozymes in bone tissue. We examined the expression and phorbol ester sensitivity of PKC isozymes in osteoblasts, bone-forming cells which can also promote osteoclast-mediated bone resorption. Normal mouse osteoblasts and 7 osteoblastic cell lines were screened by Western blotting using isozyme-specific anti-PKC antibodies. Western blotting was also employed to determine the sensitivity of the isozymes to long-term phorbol ester treatment. The results of these studies will provide important information that will aid in clarifying the role of PKC in bone remodeling.

BODY

EXPERIMENTAL METHODS

Isolation of normal mouse osteoblasts

Normal mouse osteoblasts were isolated enzymatically under sterile conditions from the calvariae of 5-6 day old CD-1 mice (19). Frontal and parietal bones were removed and incubated in 10 ml HEPES buffer (25 mM HEPES, 10 mM $NaHCO_3$, 100 mM NaCl, 3 mM K_2HPO_4, 60 mM sorbitol, 1 mM $CaCl_2$, 5 mg/ml glucose, 1 mg/ml BSA, 5-15 mg/ml collagenase (Cooper Biomedical, Sigma)) at 37°C in a shaking water bath. After each of six 20 minute incubation periods, the buffer was decanted and filtered through 35 μM mesh nylon net. Fraction 1 was discarded; fractions 2-6 were washed, pooled, and resuspended at a concentration of 0.5-1 x 10^6 cells/ml in DMEM supplemented with 15% heat-inactivated horse serum, 100 U/ml K-penicillin G, and 10 U/ml Na-heparin. The cell suspension was added to culture dishes, and the cells were allowed to attach overnight. The medium was changed the following day to remove unattached cells.

Cell Culture

UMR-106 rat osteoblastic osteosarcoma cells (American Type Culture Collection, Rockville, MD) were grown in 75 cm^2 cell culture flasks at 37°C in a humidified 5% CO_2 atmosphere in DMEM supplemented with 15% heat-inactivated horse serum and 100 U/ml K-penicillin G. The ROS 17/2.8 and 24/1 rat osteoblastic cell lines were cultured as previously described (20). Cells were passaged every 5-7 days with medium changes every 3 days.

The four human osteoblastic cell lines, MG-63, G-292, SaOS-2, and HOS-TE85, (American Type Culture Collection) were cultured under conditions recommended by the supplier.

Experimental protocols

Isozyme expression in osteoblastic cells

The growth conditions and cell densities of the osteoblast populations used for this study were equalized to the greatest extent possible prior to and at the time of harvest, respectively. The normal osteoblasts and the osteoblastic cell lines were approximately 50% confluent when harvested.

Determination of the sensitivity of PKC isozymes to long-term phorbol ester treatment

Normal mouse osteoblasts and the UMR-106 rat cell line were chosen for these studies. After isolation, the primary mouse osteoblasts were seeded in 10 cm dishes in 10 ml of culture medium. The medium was changed both the first and second days after isolation. On the third day after isolation, phorbol treatments were initiated. Phorbol 12, 13-dibutyrate (PDB, Sigma) or an equal volume of DMSO (vehicle) was added directly to the culture medium to give a final concentration of 1 μM; the contents of the dishes were mixed by swirling. Cells were treated with vehicle or PDB for 1, 3, 6, 12, 24, or 48 hr.

Experiments with the UMR-106 cells were carried out as described for the normal mouse osteoblasts except that phorbol ester treatments were initiated the day after the UMR-106 cells

were seeded (1.5 x 10^6 cells/dish) in the 10 cm dishes.

Western blotting

Cells were removed from culture dishes by scraping in RIPA buffer (phosphate buffered saline (PBS), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 0.15-0.3 TIU/ml aprotinin, 1 mM sodium orthovanadate). The cell suspension was transferred to a microfuge tube using a syringe fitted with a 21 gauge needle. The dish was washed once with additional RIPA buffer, and this was combined with the first lysate. The total volume of lysate was then passed through a 21 gauge needle approximately 15X to shear the DNA. After a 30 minute incubation on ice, additional PMSF (0.1 mg/ml) was added to each lysate. The samples were microfuged for 20 min at 4°C; the resulting supernatant was taken as the total cell lysate. An aliquot of the supernatant was removed for determination of protein, which was measured by the method of Lowry (21). The remaining supernatant was mixed with an equal volume of stop solution (17% glycerol, 8.7% (v/v) 2-mercaptoethanol, 5% SDS, 0.2 M Tris-HCl (pH 6.7), 0.1 mg/ml bromophenol blue) and boiled for 2 min. Samples were stored at -20°C until use.

For immunoblotting (22), extracts were subjected to SDS polyacrylamide electrophoresis using 5% stacking gels and 10% separating gels (0.05 amp/gel, 4-5 hours) followed by electrophoretic transfer of proteins to nitrocellulose (0.2 μM, Schleicher & Schuell) (30 volts, overnight). After transfer, membranes were blocked in Blotto B (Tris-buffered saline, 0.05% Tween-20, 1% BSA, 1% Carnation non-fat dry milk) for 1 hour at room temperature. Blots were then incubated with isozyme-specific anti-PKC antibodies (rabbit polyclonal antibodies raised against C-terminal peptides, Santa Cruz Biotechnology) for 45 minutes at room temperature on a rocking platform. Membranes were washed two times in TBS-T and subsequently incubated with goat anti-rabbit, peroxidase-conjugated secondary antibody (Sigma) for 30 minutes at room temperature on a rocking platform. After washing the membrane 3 times in TBS-T and once in TBS, immune complexes were visualized by enhanced chemiluminescence (ECL, Amersham) using Kodak X-OMAT LS film. Immune complexes were quantified by densitometry using a Biorad Model GS-670 densitometer.

In some experiments, the membranes were stripped and reprobed with a different anti-PKC antibody. Stripping was done by immersing membranes in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7)) for 30 min at 50°C in a shaking water bath. After stripping, the membranes were washed 2X for 10 min in TBS-T. Following these washes, membranes were handled as described above, starting at the point of blocking. Membranes were not stripped more than twice.

RESULTS

PKC isozyme expression in normal osteoblasts and osteoblastic cell lines

To determine if there is a characteristic pattern of PKC isozyme expression in osteoblasts, the isozyme profile of 8 different osteoblasts/osteoblast-like cell lines was elucidated by Western blotting. The osteoblasts screened included normal neonatal mouse osteoblasts, three rat osteoblastic cell lines (UMR-106, ROS 17/2.8, ROS 24/1), and four human osteoblastic cell lines (MG-63, G-292, SaOS-2, HOS-TE85).

Figure 1 shows the Western blots obtained utilizing antibodies to the conventional PKC isozymes, α, β_I, β_{II}, and γ. PKC-α and -β_I were expressed in each of the osteoblasts examined; PKC-β_{II} was detected in all but the ROS 24/1 rat line. The γ isozyme, however, was not detectable in any of the osteoblasts screened.

The novel isozymes examined were PKC-δ, -ϵ, η, and -θ (Figure 2). Like the conventional α and β_I isozymes, PKC-ϵ was expressed in each of the osteoblasts screened. The other novel isozymes, however, show more varied expression between cell lines. PKC-δ was detectable in the normal mouse osteoblasts, the UMR-106 rat line, and faintly in the SaOS-2 and HOS-TE85 human lines. PKC-η was also expressed in the normal mouse osteoblasts, the UMR-106, SaOS-2 and HOS-TE85 cells as well as in the ROS 24/1 and MG-63 lines. PKC-θ was expressed only in the SaOS-2 and HOS-TE85 lines; in some experiments, θ was also detected in the ROS 24/1 line (data not shown).

Expression of the atypical ζ and ι isozymes was also determined for the osteoblasts. Because PKC-ζ and -ι share a common C-terminus (3), the C-terminal PKC-ζ antibody cross reacts with PKC-ι, and both isozymes are seen on the same blot. PKC-ζ is detected as a single band at approximately 75 kilodaltons (kDa) and PKC-ι as a single band at approximately 65 kDa. As shown in Figure 3, both isozymes were expressed in each of the osteoblasts screened. The highest molecular weight band (80 kDa) seen on this blot is PKC-α; cross-reactivity of C-terminal ζ antibodies with PKC-α has been reported previously (23). As was observed with the PKC-α-specific antibody (Figure 1), this isozyme was expressed in all of the osteoblasts screened.

In each of the osteoblasts screened, only one band was observed for the α, β_{II}, δ, θ, ζ, and ι isozymes (Figures 1-3). In contrast, blots for the β_I, ϵ, and η isozymes (Figures 1, 2) revealed two distinct bands of slightly different molecular weights in some cell lines. The β_I isozyme was detected as a doublet in the normal osteoblasts and all of the cell lines except the human G-292 line, which exhibited only the higher molecular weight band (Figure 1). The ϵ isozyme was also detected as either a singlet or a doublet, depending on the cell line (Figure 2). Most of the osteoblasts screened exhibited only one band for ϵ, but the normal osteoblasts and the ROS 17/2.8 line exhibited two bands; the second band occurred at a slightly lower molecular weight than the primary band observed in these and the other osteoblasts. PKC-η was detected as a single band in the normal mouse osteoblasts and the rat lines (Figure 2), but appeared as a doublet in the three human lines in which it was expressed; the second band had a greater apparent molecular weight than the singlet observed in the mouse and rat lines. It is likely that these doublets represent different phosphorylation forms of a given isozyme, as has been reported previously (2).

Sensitivity of PKC isozymes to long-term phorbol ester treatment

Normal mouse osteoblasts and the UMR-106 rat osteoblastic cell line were chosen for these studies. After treatment with either a vehicle control (DMSO) or 1 μM phorbol 12,13-dibutyrate (PDB) for 1, 3, 6, 12, 24, or 48 hr, cells were harvested and Western blots conducted utilizing isozyme-specific anti-PKC antibodies.

Conventional isozymes

As shown in Figure 4, the conventional α and β_I isozymes were only moderately sensitive to long-term phorbol ester treatment. In the normal osteoblasts, slight down-regulation of PKC-α was detectable after only a 1 hr treatment with PDB (1 μM) (77% of 1 hr control, Figure 5; all % of control calculations are based on time-matched controls). Down-regulation of PKC-β_I was evident at 3 hr, having decreased to 64% of control (Figure 5). At 6 hr, both PKC-α and -β_I had fallen to 48% of control levels. Down-regulation of both isozymes was maintained at approximately 50% at all later time points examined.

Similar effects of long-term PDB treatment on the α and β_I isozymes were observed in the UMR-106 line (Figure 4). Down-regulation of PKC-α and -β_I was first detectable following 3 hr treatment with PDB (1 μM); at 3 hr, α and β_I were decreased to 77% and 85% of control, respectively (Figure 5). At 6 hr, both isozymes were down-regulated to a slightly greater extent, falling to 65-70% of control levels. PKC-α continued to decline to 42% and 24% of control at 12 and 24 hr, respectively, but then increased slightly (33% of control) at 48 hr. Levels of PKC-β_I also continued to decline with longer treatment periods, dropping to 64%, 35%, and 19% of control at 12, 24, and 48 hr, respectively (Figure 5).

Novel isozymes

In contrast to the conventional isozymes, the novel PKC-δ and -ϵ isozymes were highly sensitive to long-term phorbol ester treatment, especially in the normal mouse osteoblasts (Figure 6). In the normal osteoblasts, PKC-δ was dramatically down-regulated after 1 hr PDB treatment (55% of control, Figure 7) and further down-regulated at 3 hr (24% of control). In examining the Western blot, PKC-δ appears to be completely down-regulated at all subsequent time points examined. The densitometric values, however, suggest that there is substantial immunoreactivity remaining at these later time points. It seems likely that these values are overestimated though, due to variations in the background of the blot. PKC-ϵ was also completely down-regulated by long-term phorbol treatment (Figure 6), although the time course was somewhat different than for δ. Down-regulation of PKC-ϵ was first apparent at 6 hr and was complete at this time point (Figures 6, 7). Like PKC-δ, down-regulation of PKC-ϵ was maintained at all subsequent time points.

Expression of the δ and ϵ isozymes was affected in a parallel manner by long-term PDB treatment (1 μM) in the UMR-106 osteoblastic line (Figures 6, 7). PKC-δ was partially down-regulated after 1 hr PDB treatment (71% of control), further down-regulated at 3 and 6 hr (17% and 5% of control, respectively), and completely down-regulated at all subsequent time points. Down-regulation of PKC-ϵ was first detectable in the UMR-106 cells at 3 hr (71% of control), and declined progressively with 6, 12, and 24 hr treatments (40%, 21%, and 11% of control, respectively). PKC-ϵ isozyme levels reached a plateau between 24 and 48 hr treatment.

The novel η isozyme was unique in that it was affected by phorbol ester treatment in two

distinct ways. First, in both normal osteoblasts and UMR-106 cells, phorbol treatment caused a slight decrease in the mobility of PKC-η (Figure 6). This affect, seen as a slight upward shift in the PKC-η band, was apparent at all time points examined and may indicate that η was phosphorylated with phorbol treatment. Second, phorbol treatment caused a down-regulation of the η isozyme (Figures 6, 7), as described above for the other isozymes. In the normal osteoblasts, PKC-η was only moderately sensitive to long-term PDB treatment. Down-regulation was apparent at 6 hr (75% of control) but failed to progress with longer treatment periods. In contrast, in the UMR-106 line, down-regulation of η was first detectable at 3 hr (88% of control) and declined further at later time points, falling to 82%, 29%, and 16% of control levels at 6, 12, and 24 hr, respectively. PKC-η levels reached a plateau between 24 and 48 hr treatment.

Atypical isozymes

Unlike the conventional and novel PKC isozymes, the atypical ζ and ι isozymes were insensitive to long-term phorbol ester treatment at all time points examined (Figures 8, 9). This insensitivity was observed in both the normal mouse osteoblasts and the UMR-106 cell line.

As seen in Figure 8, the uppermost PKC-α band observed in blots with the anti-PKC ζ/ι antibody was down-regulated by phorbol ester treatment. In the normal osteoblasts, down-regulation of α was detectable after 1 hr PDB treatment (69% of control, Figure 9). PKC-α levels were slightly higher at 3 hr but were still lower than in control cells (81% of control). At 6 hr, PKC-α levels fell to 38% of control, and remained at 45-60% of control at all subsequent time points. In the UMR-106 cells, down-regulation of PKC-α was apparent after 3 hr PDB treatment (87% of control, Figure 9). Levels of this isozyme declined progressively at later time points falling to 82%, 50% and 39% of control at 6, 12, and 24 hr, respectively. The down-regulation observed at 24 hr was maintained with 48 hr PDB treatment (35% of control). These results are consistent with those obtained using the PKC-α-specific antibody (Figures 4, 5).

DISCUSSION

The current results indicate that there is a characteristic pattern of PKC isozymes seen in osteoblasts across species and in both normal and osteosarcoma-derived cells. The PKC isozymes found to be present in all of the osteoblasts screened were PKC-α, -βI, -ϵ, -ζ, and -ι. PKC-βII was expressed in each of the osteoblasts except the ROS 24/1 rat line, which is a less differentiated osteoblastic cell (24). The novel δ, η, and θ isozymes were detectable only in some of the osteoblasts examined. This variation is not due to species specificity of the anti-PKC antibodies since PKC-η, for example, is detectable in normal mouse osteoblasts as well as select rat and human osteoblast-like cell lines. The variability in expression of these isozymes, like PKC-β_{II}, may be a reflection of the different stages of differentiation of the osteoblasts screened (24-29). Conversely, the variable expression of PKC-δ, -η, and -θ, may be an indication that these isozymes are not important for the basic osteoblastic functions of these cells.

This the first characterization of the isozymes present in osteoblastic cells, and indicates the differences between this tissue and others, such as brain, which expresses all the known isozymes of PKC (1) and kidney which expresses PKC-α, -δ, -ϵ, and -ζ (2). The α, β_I/β_{II}, δ, ϵ, and ζ isozymes are ubiquitously expressed (2), and of these, all but PKC-δ are expressed in osteoblasts. None of the osteoblasts screened express PKC-γ. This result is not surprising, however, as this isozyme has been detected only in the central nervous system (1). A broad determination of the tissue expression of the other isozymes examined in this study, PKC-η, -θ, -ι, has not yet been carried out. Initial studies suggest that PKC-η and -θ have a rather limited distribution, with η being expressed predominantly in the skin and lung (30,31) and θ largely in skeletal muscle (32). Both isozymes are expressed at a lower extent in the brain and spleen.

As expected, there were differences in phorbol ester sensitivity among the different osteoblast PKC isozymes. The conventional and novel isozymes were down-regulated by long-term phorbol ester treatment whereas the atypical isozymes were not. These results are consistent with the ability of these classes to bind phorbol esters. The conventional and novel isozymes possess the two zinc finger motifs in the conserved C1 domain of PKC necessary to bind DAG and phorbol esters (2); atypical isozymes lack one of the two zinc fingers, however, and are unable to bind DAG or phorbol esters (2,4).

Down-regulation occurred quite rapidly for nearly all of the phorbol-sensitive osteoblast isozymes. All of the sensitive isozymes, with the exception of PKC-η in normal osteoblasts, were significantly diminished with 6 hr phorbol treatment. This rapid time course of down-regulation has implications for experiments in which the phorbol ester is used as a tool to presumably activate PKC. Unless the exposure times are very short, the results may actually be due to a down-regulation, with an opposite interpretation for the role of PKC in the process. Another possibility in such experiments is that the initial activation, rather than the subsequent down-regulation of the kinase activity, is the critical event. Probably the clearest interpretation is in experiments in which the PKC is first down-regulated, and then a short-acting stimulus is added.

Although both the conventional and novel isozymes were down-regulated by long-term phorbol treatment in osteoblasts, the extent of the down-regulation observed for these two classes was somewhat different. The conventional α and β_I isozymes tended to be less sensitive to long-term phorbol ester treatment than the novel isozymes, particularly PKC-δ and -ϵ. This

difference in sensitivity is not likely to be related to the exposure times for the blots; even if the blots for PKC-δ were exposed for long periods of time, it was still not possible to detect bands at the later time points (data not shown).

Differences in the extent of phorbol-induced down-regulation of conventional and novel isozymes has been reported previously (33). The reason for this difference is not clear. Recently, however, a correlation has been noted between the ability of specific PKC isozymes to down-regulate and their ability to induce vesicle traffic (34). By inducing vesicle traffic, these isozymes promote their own destruction; that is, the traffic carries the PKC for sorting and degradation. It is possible that the novel isozymes are better able to induce such traffic than the conventional isozymes, thus accounting for the difference in the extent of down-regulation observed for these classes.

No major differences in phorbol sensitivity are apparent in comparing the results obtained in the normal osteoblasts and the UMR-106 cell line. All of the isozymes in normal cells that are sensitive to phorbol ester treatment are also sensitive in the UMR-106 cells. There are differences as to the extent of down-regulation observed in the two cell types; for example, η appears to be down-regulated to a greater extent in the UMR-106 cells than in the normal osteoblasts. PKC-η undergoes a change in mobility and down-regulation in both cell types, however, illustrating that the same pattern of response is observed in both osteoblasts.

As mentioned above, the failure of long-term phorbol treatment to down-regulate the atypical ζ and ι isozymes is consistent with the inability of these isozymes to bind phorbol ester (2,4). A similar insensitivity of PKC-ζ to phorbol has been described in other cell types, including Jurkat T lymphoma cells (35) and renal mesangial cells (36). Phorbol-induced down-regulation of ζ has also been reported (37), but in at least some of these studies, the authors have employed the same PKC-ζ antibody used in our studies (33). For example, in a recent study carried out in urinary tract smooth muscle cells, the anti-PKC-$\zeta/\iota/\alpha$-antibody was utilized to examine the sensitivity of the ζ isozyme to 24 hr phorbol myristate acetate (PMA) treatment. As in our studies, the Western blot with this antibody revealed three bands at approximately 80, 75, and 65 kDa. The 80 kDa ζ-reactive band was down-regulated by 24 hr PMA treatment, but the other two bands were unaffected by this treatment. The 80 kDa band was taken to be PKC-ζ, and no mention was made of the other two bands observed in this blot. Therefore, when results obtained with this antibody or other C-terminal PKC-ζ antibodies are interpretted, the ability of this antibody to cross-react with the phorbol-sensitive PKC-α isozyme must be considered.

CONCLUSIONS

The studies carried out thus far demonstrate that osteoblasts have a characteristic PKC isozyme profile, including both phorbol ester-sensitive and insensitive isozymes. Down-regulation of sensitive PKCs is detectable within 6 hr of phorbol treatment. This time course and the presence of phorbol-insensitive isozymes in osteoblastic cells must be considered in interpreting the effects of phorbol esters on bone remodeling. The results of these studies provide important information which will aid in clarifying the role of PKC in bone remodeling.

In addition, the results of these studies provide a solid basis for the ongoing studies to examine the effects of 17β-estradiol, tamoxifen, and additional sex steroids on PKC isozyme expression. Now that we have a clear picture of the pattern of isozyme expression expected in an osteoblast/osteoblast-like cell, we can focus our efforts on exploring the possible regulation of these isozymes. Modulation of one or more of these "osteoblastic" isozymes by estrogen or tamoxifen is likely to have functional consequences for the osteoblast.

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APPENDIX 1
Figures illustrating the expression and phorbol-ester sensitivity of PKC isozymes in osteoblasts

Comments on figures:

Figures 1-3: These figures show the results of the experiments carried out to determine which PKC isozymes are expressed in osteoblasts/osteoblast-like cells. The figures are Western blots done with isozyme-specific anti-PKC antibodies. Figure 1 shows the expression of the conventional isozymes, Figure 2 the novel isozymes, and Figure 3 the atypical isozymes (and PKC-α). From left to right in each figure are:

1) MBrStd: Mouse brain standard (used as a positive control)
2) Normal OB: Normal mouse osteoblasts
3) UMR-106, ROS 17/2.8, ROS 24/1: Rat osteoblastic cell lines
4) MG-63, G-292, SaOS-2, HOS-TE85: Human osteoblastic cell lines

Figures 4-9: These figures show the results of the experiments carried out to determine the phorbol ester-sensitivity of the PKC isozymes expressed in normal mouse osteoblasts and the UMR-106 rat cell line.

Figures 4, 6, and 8 are the Western blots done with the isozyme-specific antibodies. The left panels in each figure are from the normal osteoblasts and the right panels from the UMR-106 cells. The first lane in each blot is an untreated control (Untrt C), and the remaining lanes show the time course of vehicle (C) and phorbol ester (P) treatment, which include 1, 3, 6, 12, 24, and 48 Hr time points.

Figures 5, 7, and 9 are the results of the densitometric analysis of the blots shown in Figures 4, 6, and 8, respectively. The label for the Y axis in each graph is % of control; that is, the phorbol ester-treated samples at each time point are taken as a percent of the control sample at the corresponding time point (time-matched controls).

Figure 1

PKC Isozyme Expression in Osteoblasts
Conventional PKC's

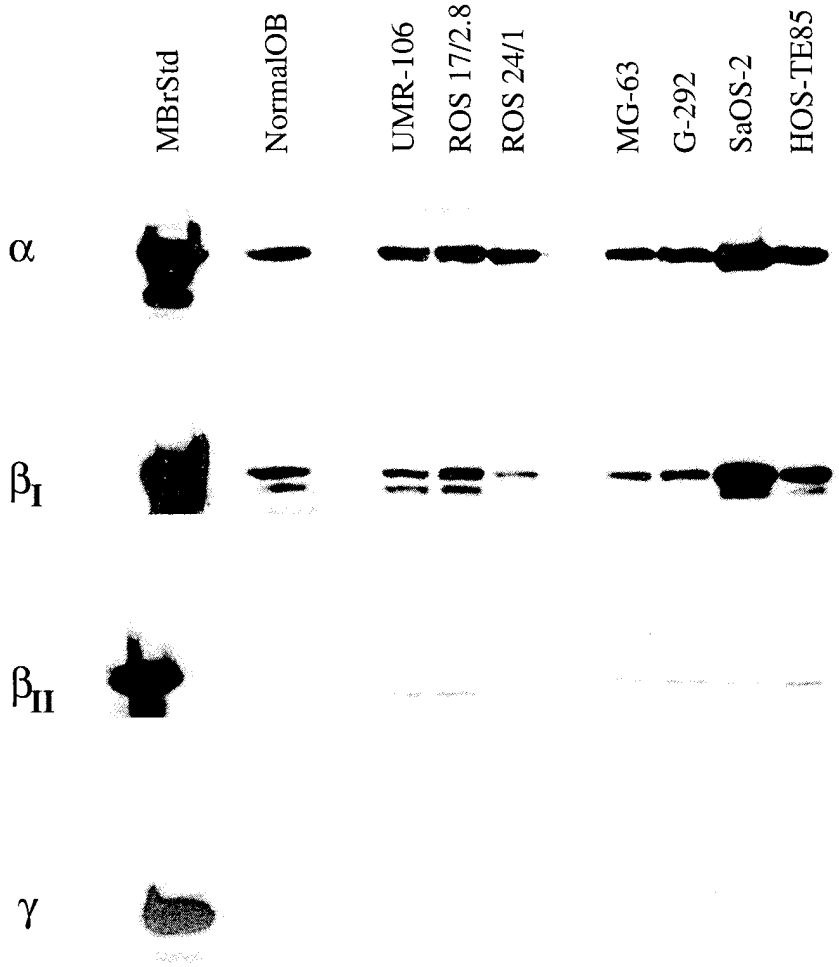


Figure 2

PKC Isozyme Expression in Osteoblasts
Novel PKC's

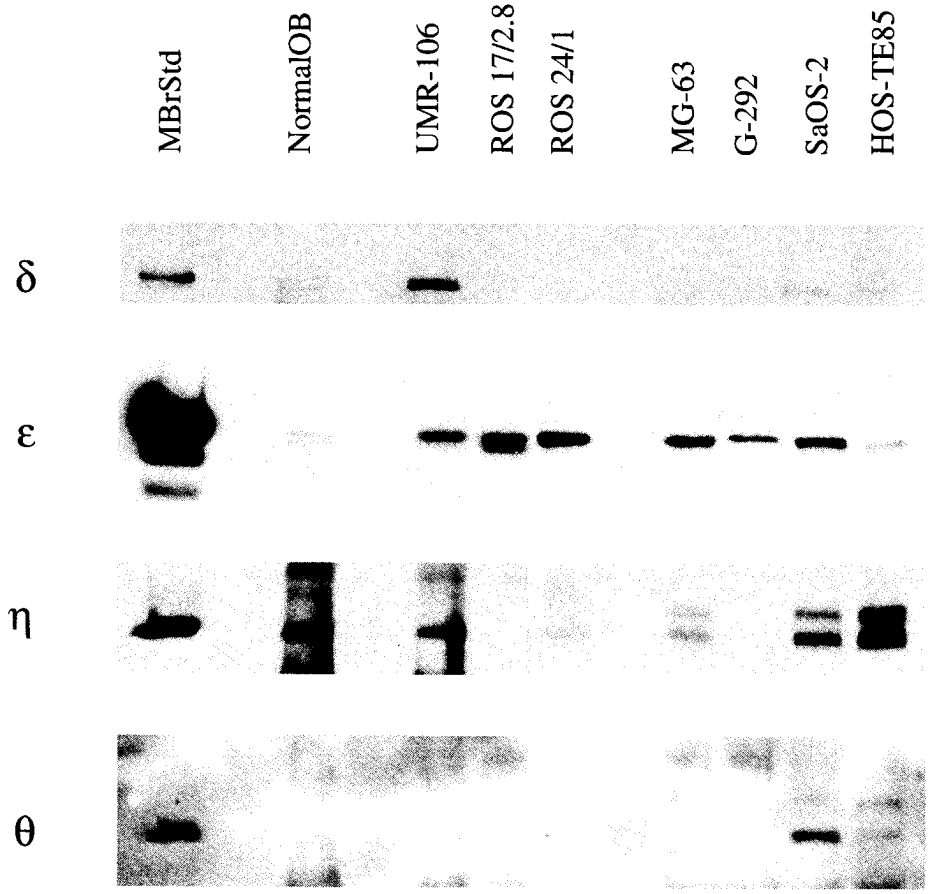


Figure 3

PKC Isozyme Expression in Osteoblasts
Atypical PKC's and PKC-α

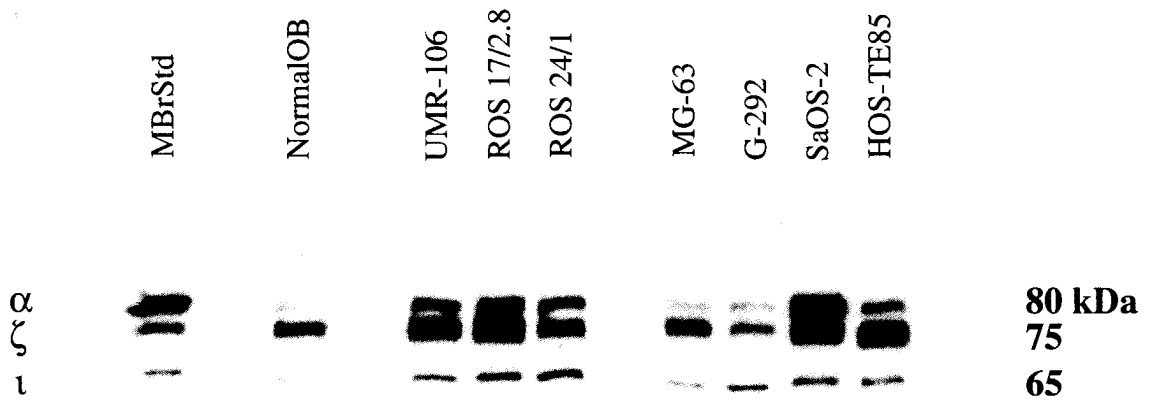


Figure 4

Time Course of Phorbol Ester-Induced Down Regulation: Conventional PKC's

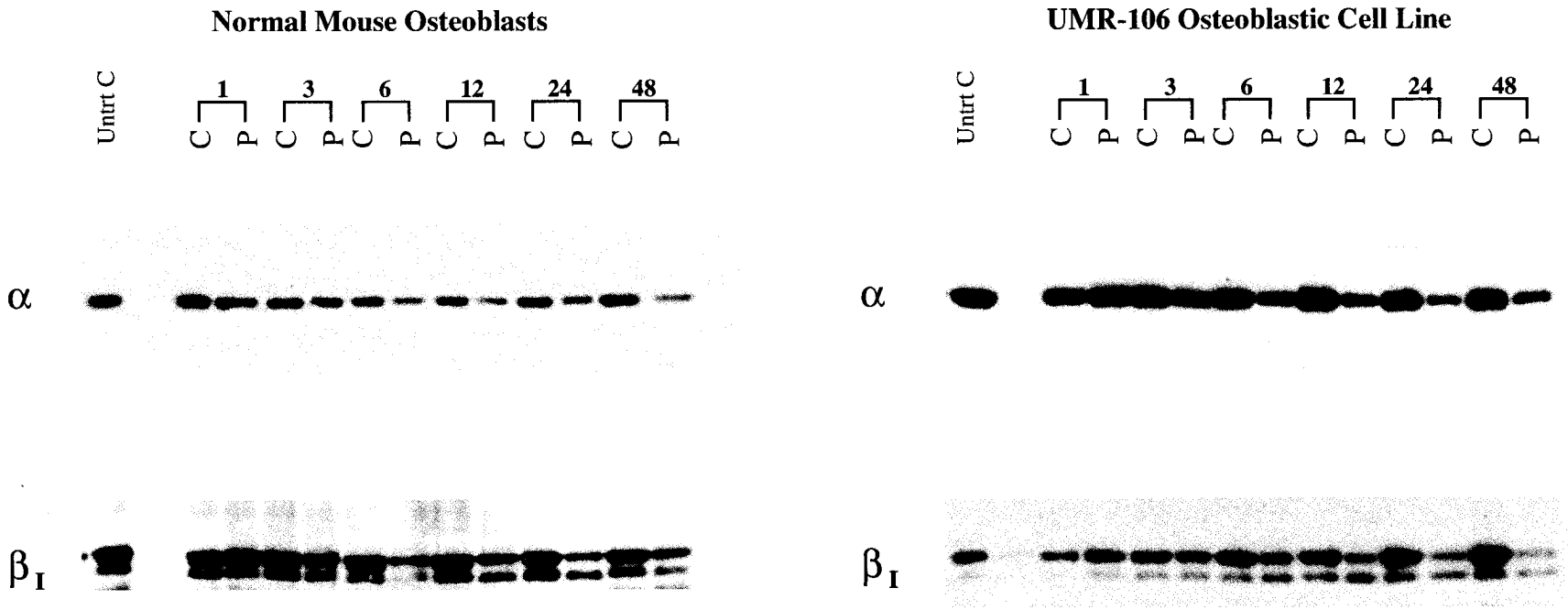


Figure 5

Down-Regulation of Conventional PKC Isozymes
Densitometric Analysis of Blots

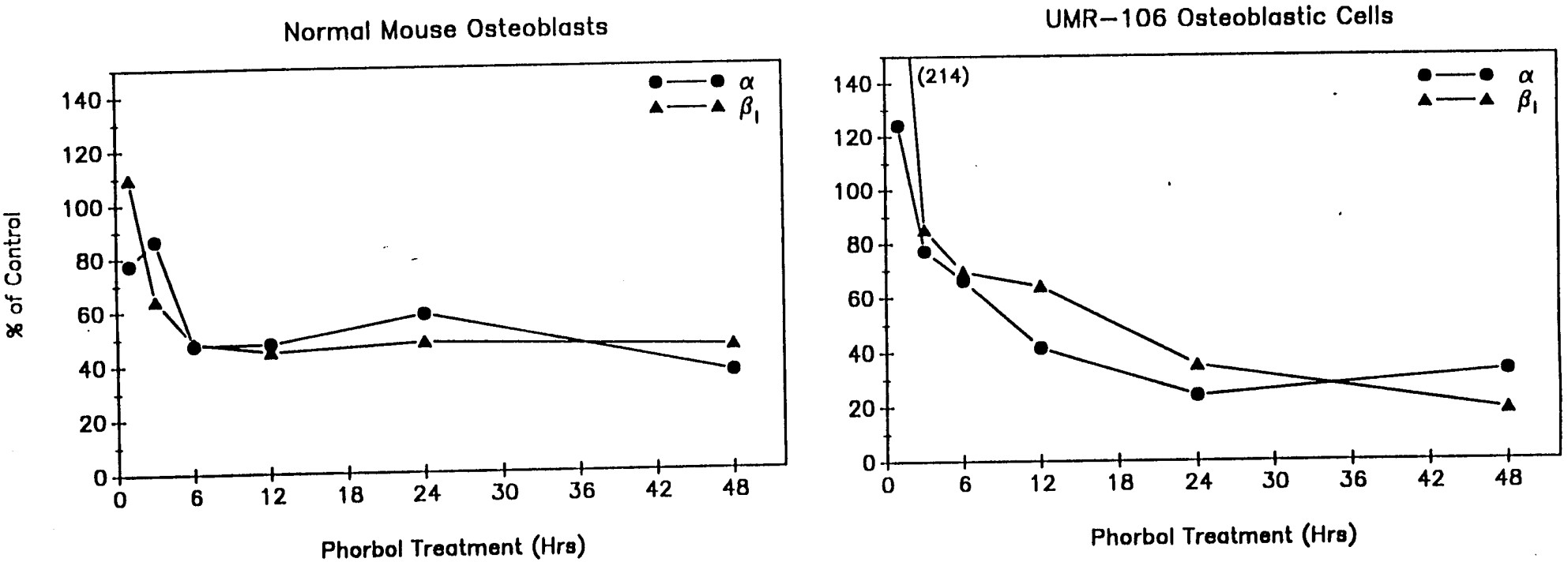


Figure 6

Time Course of Phorbol Ester-Induced Down Regulation: Novel PKC's

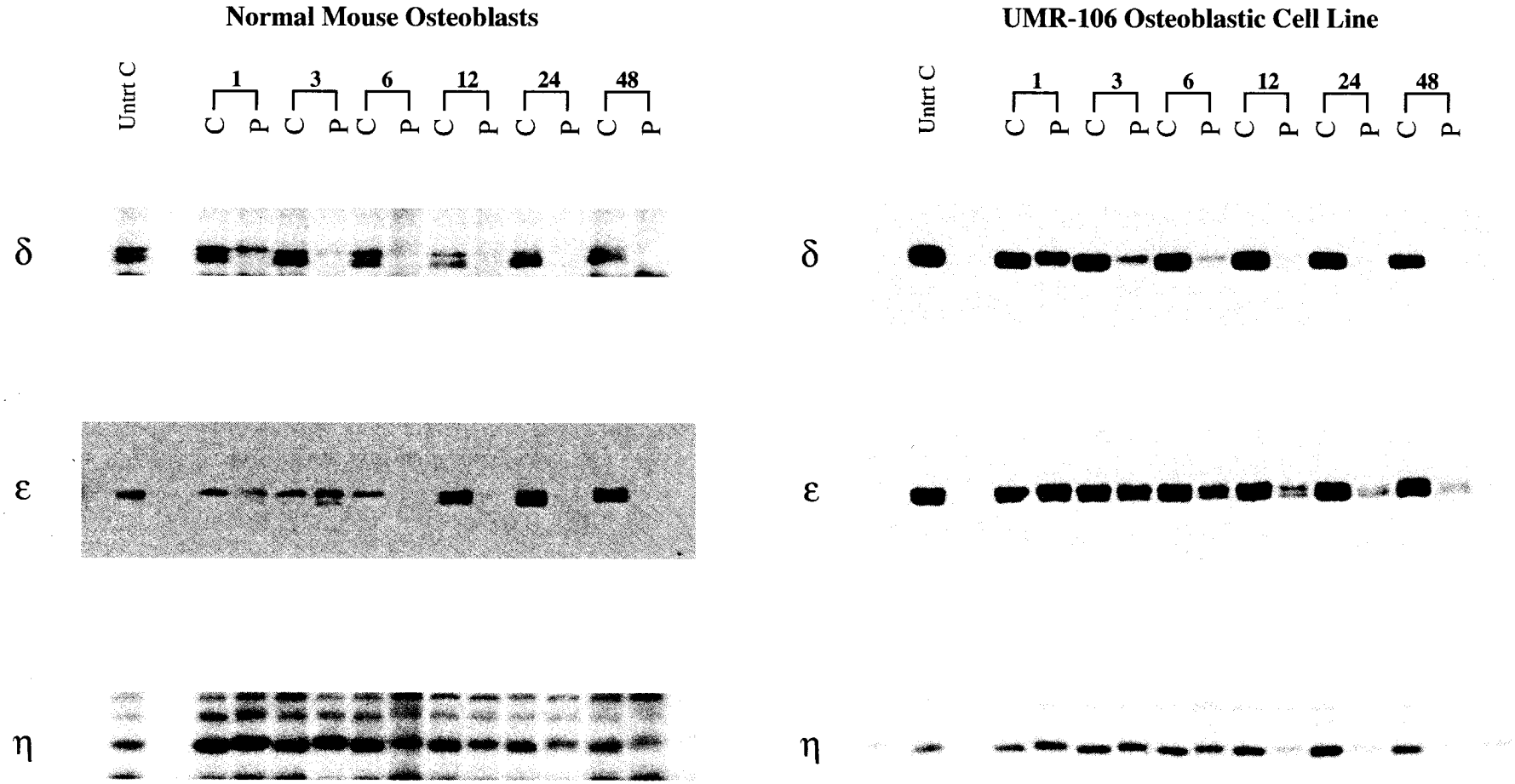
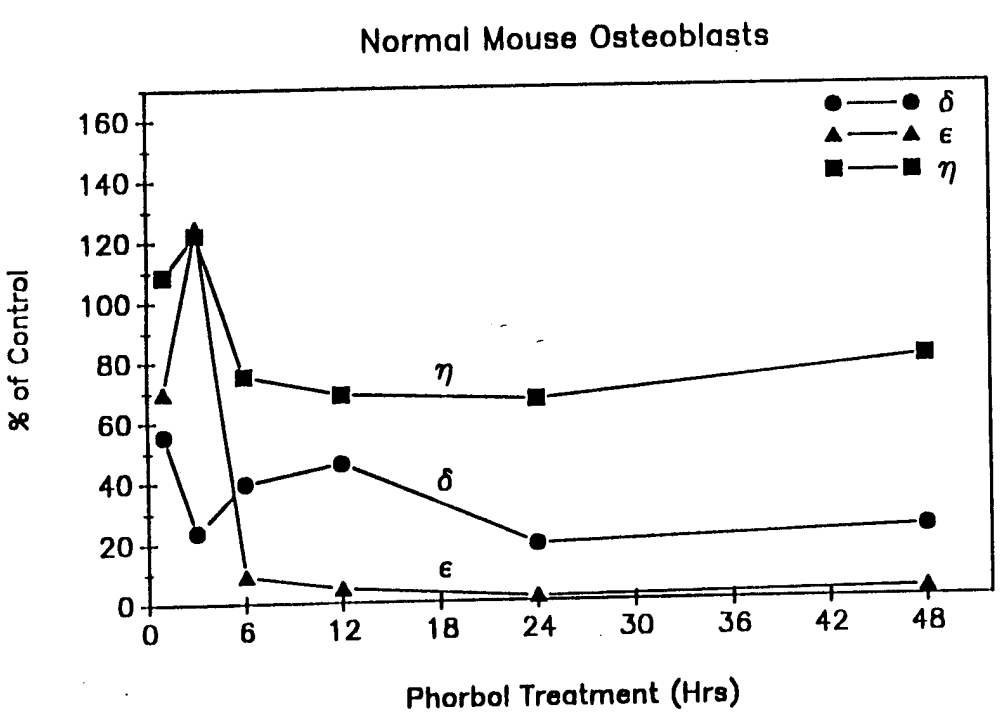


Figure 7

Down-Regulation of Novel PKC Isozymes
Densitometric Analysis of Blots



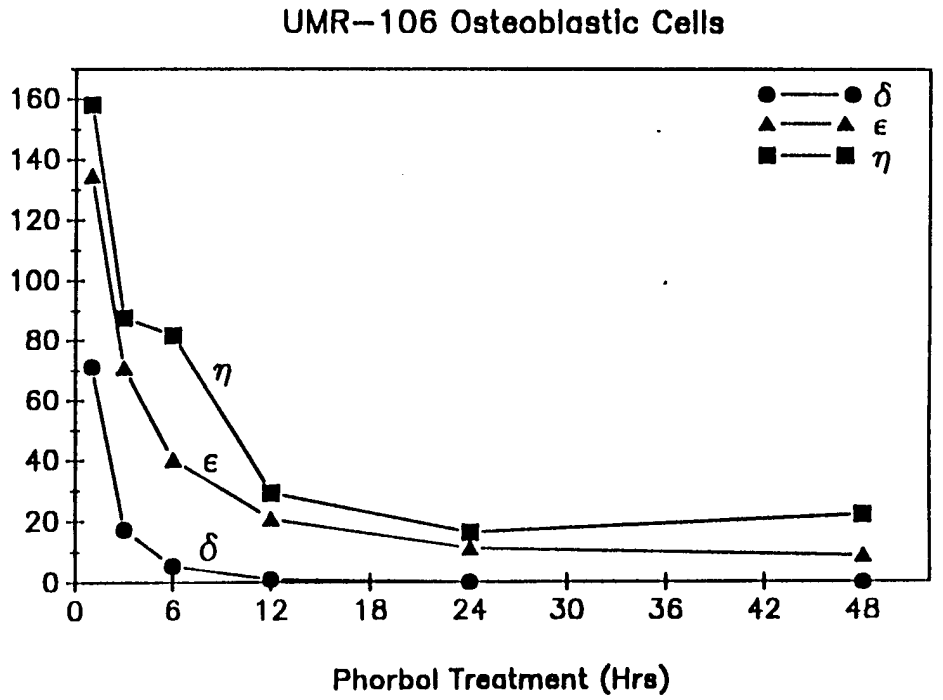


Figure 8

Time Course of Phorbol Ester-Induced Down Regulation: Atypical PKC's and PKC-α

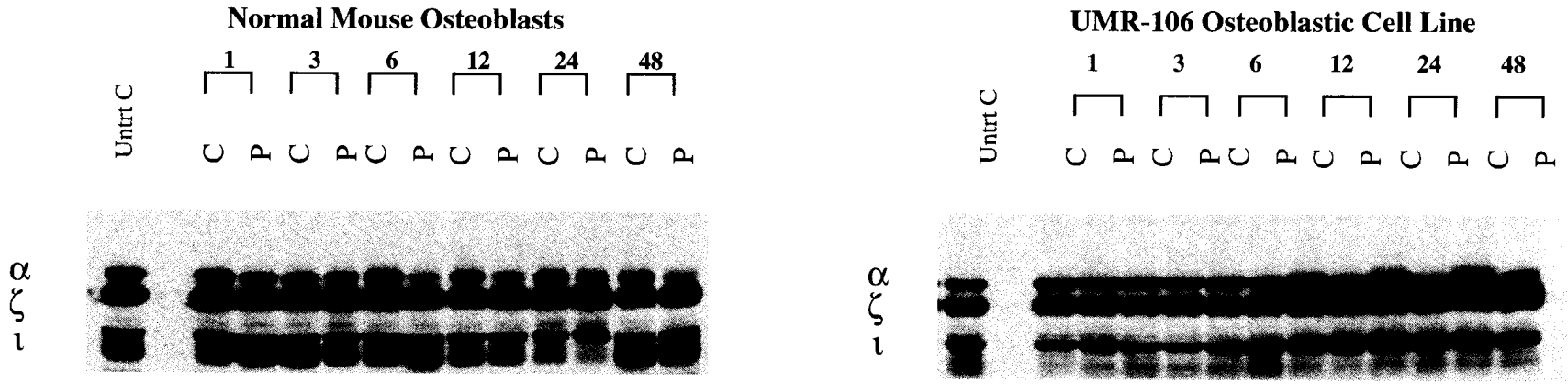
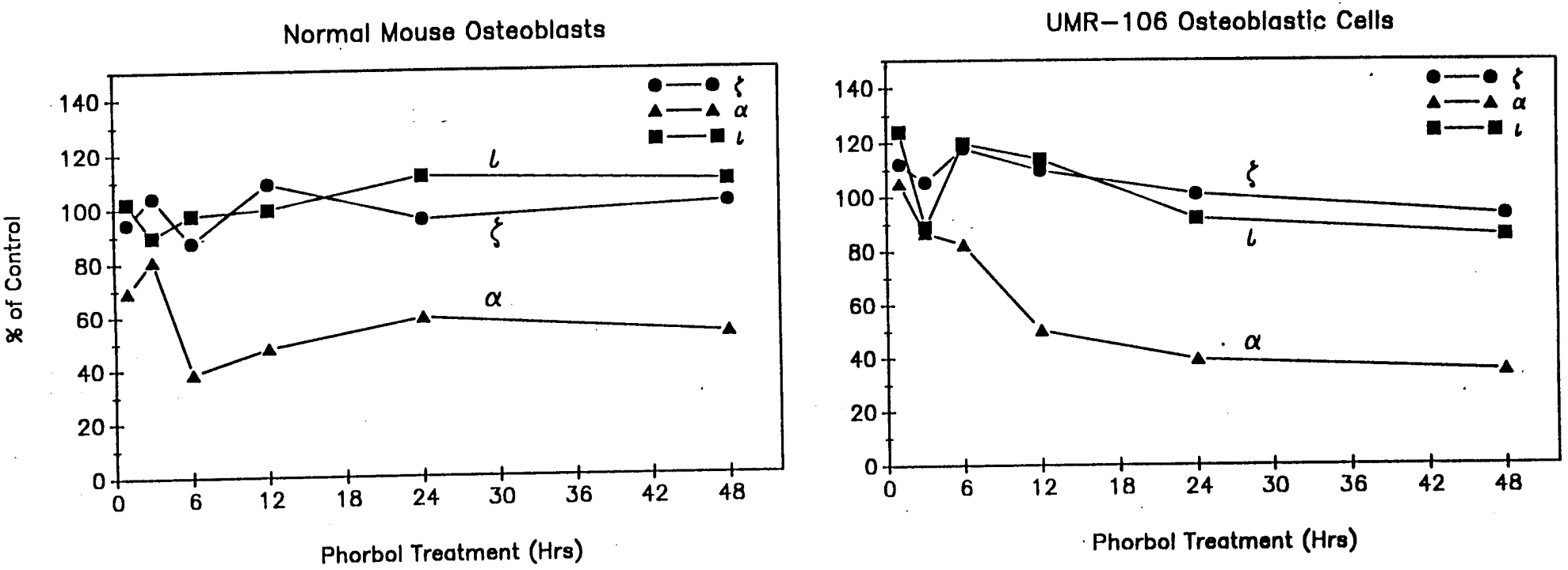


Figure 9

Lack of Down-Regulation of Atypical PKC Isozymes
Down-Regulation of PKC-α
Densitometric Analysis of Blots



APPENDIX 2
Additional Data

Effect of 17β-estradiol treatment on PKC isozyme expression in UMR-106 osteoblastic cells

Ikegami *et al.* (Endocrinology 135:782-789, 1994) demonstrated that estrogen receptor (ER) expression is increased in HOS-TE85 human osteoblastic cells following their release from G_1/S phase synchronization; the greatest increase in estrogen receptor expression was seen 6 hr following release. Based on these studies, we designed an experimental protocol in which UMR-106 cells were synchronized, released from synchronization, and at 6 hr post-release treated with either vehicle or 17β-estradiol. At the end of the treatment period, cells were harvested for Western blotting to examine PKC isozyme expression. By employing this protocol, we hoped to treat the UMR-106 osteoblastic cells at a time when ER expression was maximal. This would then allow us to detect effects on isozyme expression which may otherwise be masked due to the presence of mixed populations of cells; that is, cells in various stages of the cell cycle.

UMR-106 cells were synchronized at the G_1/S phase boundary by intermittent exposure to thymidine and hydroxyurea, as described by Ikegami *et al.* 6 hr after releasing cells from synchronization, treatment with either vehicle (absolute ethanol) or 1 nM 17β-estradiol was initiated and continued for either 3, 6, 12, 18, or 24 hr. Western blots with an anti-PKC α/β antibody (mouse monoclonal, Amersham) revealed an estrogen-sensitive protein; following 24 hr 17β-estradiol treatment, the level of this protein was dramatically increased relative to control (Figure 10, showing 12, 18, and 24 hr time points).

This estrogen-sensitive protein does not appear to be either PKC-α or -β; PKC-α and -β both have molecular weights of approximately 77-80 kDa, but the single, estrogen-sensitive band detected has a molecular weight of 120-200 kDa. In additional studies, we determined that this 120-200 kDa protein was insensitive to long-term phorbol ester treatment (data not shown). It is possible that the estrogen-sensitive protein detected is PKC-μ. This recently identified isozyme has an apparent molecular weight of approximately 115 kDa (Reference 5, above), which is much higher than the molecular weights of all other known PKC isozymes. In addition, studies in transfected HeLa cells demonstrated that long-term phorbol ester treatment did not cause down-regulation of PKC-μ (Reference 6, above). Additional studies will be carried out to identify this estrogen-sensitive protein.

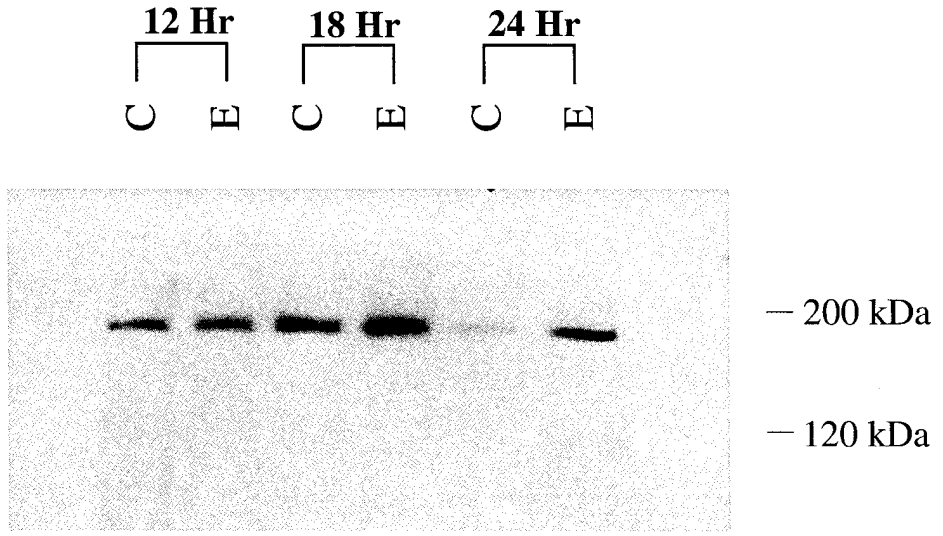
Effect of tamoxifen on PKC isozyme expression in UMR-106 osteoblastic cells

Treatment of UMR-106 osteoblastic cells with tamoxifen (1 nM, 10 nM, 100 nM, or 1 μM) for 48 hr had no effect on expression of the PKC-δ isozyme, as determined by Western blotting (Figure 11; MBrStd, mouse brain standard). The effect of these treatments on expression of the other PKC isozymes present in the UMR-106 cells will be determined. Furthermore, additional treatment periods (<48 hr, >48 hr) will be examined.

Figure 10

Time Course of 17β-Estradiol Treatment Following Release of UMR-106 Cells from Synchronization

UMR-106 Osteoblastic Cell Line



High MW Band Detected with PKC- α/β Antibody

Figure 11 48 Hr Treatment of UMR-106 Cells with Tamoxifen